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CERTIFICATE

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Dated 1 February 2000.

Neville Harris
Commissioner of Patents

Patents Form No. 4 Our Ref: JP801897

Patents Act 1953

PROVISIONAL SPECIFICATION

TRANSFORMATION AND REGENERATION OF *ALLIUM* PLANTS

We, **NEW ZEALAND INSTITUTE FOR CROP & FOOD RESEARCH LIMITED, a registered New Zealand company and Crown Research Institute under the Crown Research Institute Act 1992, of Gerald Street, Lincoln, Christchurch New Zealand** do hereby declare this invention to be described in the following statement:

PT0441664

(followed by page-1a)

Transformation and regeneration of *Allium* plants

Field of Invention

The invention relates to a method of transforming plants of the *Allium* family and more particularly to the transformation of onion plants. The invention also relates to the transformed plants.

Background of the Invention

There are no published protocols for the transformation and regeneration of *Allium* species. The *Allium* crop species are probably the most economically important vegetable species for which transformation technology is unavailable. For other major vegetable crops, confirmed transformation systems have been produced.

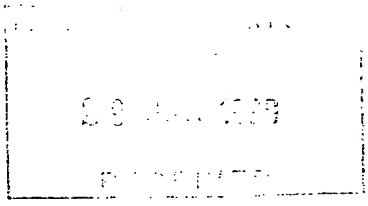
Initially, many monocotyledons were thought to be unsusceptible to *Agrobacterium*-mediated transformation. The development of direct gene transfer techniques soon led to bombardment being the favoured method of monocotyledon transformation. However, direct gene transfer is not without its problems. Often, low transformation frequencies and a high frequency of unusual integration patterns has been observed in transgenic plants. Recently, *Agrobacterium*-mediated transformation of monocotyledons has gained favour and many monocotyledonous species (including rice, wheat, barley, maize and sugarcane) have now been transformed using this method. A key component in the success of these systems has been the use of highly embryogenic tissue types, and precise

(followed by page 2)

post transformation selection protocols.

Recently, Haseloff (1997) has modified the *gfp* gene to enhance its use as a transgenic marker gene in viable plant systems. Green fluorescent protein (GFP) enables researchers to follow precisely the fate of any cells expressing this gene and so optimise post transformation cell survival. Such a system has been useful in the development of the onion transformation protocol reported here.

As monocotyledons, the *Allium* species were predisposed to be recalcitrant to transformation. Onions (*Allium cepa L*) are a crop with diverse environmental requirements. It has, therefore, been relatively understudied with respect to the application of biotechnology. There are only a few reports of DNA delivery to *Alliums* (Klein 1987; Dommisse et al. 1990; Eady et al. 1996; Barandiaran et al. 1998). Three workers used direct gene transfer whilst Dommisse et al.(1990) demonstrated that *Agrobacterium*-mediated transformation may be possible. Recently some reports of regeneration protocols for *Alliums* that are appropriate for transformation study have become available (Hong and Deberg 1995; Xue et al. 1997; Eady et al. 1998; Saker 1998). Only one report exists on the development of potential selective agents for use in *Allium* transformation (Eady and Lister 1998a). The information available is sparse. However we believe that, if the right gene constructs are delivered into the right tissue and selected using optimised selection systems then it should be possible to produce transgenic *Allium* plants.



Object of the Invention

It is therefore an object of the invention to provide a method for producing transgenic *Allium* plants or to at least provide the public with a useful choice.

In this specification we report the first repeatable protocol for the production of transgenic *Allium* plants.

Summary of the Invention

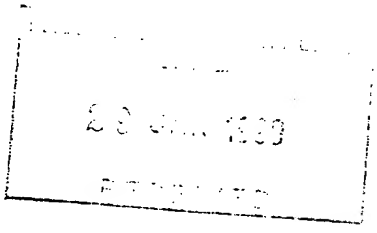
The invention provides a method of transforming *Allium* plants.

In particular, the invention provides an *Agrobacterium tumefaciens* – mediated transformation method for *Allium* plants.

Preferably the transformed plants are onions (*Allium cepa L*). More preferably the plants are the open pollinated onion cultivar Canterbury Longkeeper.

Preferably immature embryos are used as the explant source.

Preferably the embryos are transformed using a binary vector and more preferably a binary vector carrying the m-gfp5-ER reporter gene. Other binary vectors or T-DNA containing plasmids may, however, be used.



The embryos may be transformed with the *nptII* antibiotic resistance gene. However other genes may be used.

In particular, the invention provides a method of transforming *Allium* using immature embryos as an explant source, including:

a) isolating immature embryos;

b) innoculating cultures with an *Agrobacterium tumefaciens* strain containing a binary vector;

c) wounding embryos and infiltrating embryos with agrobacteria;

d) transferring embryos to geneticin and timentin;

e) culturing embryo pieces in the dark; and

f) selecting putative transgenic cultures;

g) regenerating shoots.

The invention also provides transformed *Allium* plants. Preferably the *Allium* plants are transformed using protocols in line with the method of the invention.

Brief Description of the Drawings

Embodiments of the invention are now described, by way of example only, with reference to the drawings in which:

Figure 1 shows a) GFP expression in embryo tissue after 5 days of cocultivation (x50). b) GFP expression after 2 weeks (x50). c) GFP sector after 6 weeks culture (x25). d) Independent GFP positive tissue (x5). e) GFP positive onion shoot culture (x5). f) Two GFP negative (left) and two GFP positive (right) roots from independent plants (x10). g) Transgenic onion plant (x0.2).

Figure 2 shows Southern analysis of the *gfp* gene of primary transformants: Bluescript plasmid containing the *gfp* gene (uncut), 1 copy number control (lane 1), 10 copy number control (lane 2),blank (lane 3), non transgenic onion (lane 4), 7 transgenic onion plants (lanes 5-11), bluescript plasmid containing the *gfp* gene (uncut), 1 copy number control (lane 12), 10 copy number control (lane 13), blank (lane14), non transgenic onion (lane 15), 6 transgenic onion plants (lanes 16-21).

Detailed Description of the Invention

Materials and Methods

Plant material: Field grown, open-pollinated Canterbury Longkeeper (CLK) umbels of *Allium cepa* L. were used as a source of immature embryos. Immature embryos were isolated as described by Eady et al. (1998).

Bacterial strain: *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector *pBIN m-gfp-ER* (Haseloff 1997) was used. Cultures were grown to log phase in LB media containing 50 mg/l of kanamycin and then stored at -80°C in 1 ml aliquots

containing 15% glycerol. Aliquots were used to innoculate 50 ml overnight cultures. The following morning cultures were replenished with an equal volume of LB containing antibiotic and 100 μM acetosyringone and grown for a further 4 hours. *Agrobacteria* were isolated by 10 minute centrifugation at 4500 rpm and resuspended in an equal volume of P5 (Eady and Lister 1998a) containing 200 μM acetosyringone.

Transformation procedure: Isolated immature embryos were placed in groups of 20 on P5 medium (Eady and Lister 1998a) for up to 36 h prior to transformation. Batches of 40 embryos were then transferred into 0.8 ml of *Agrobacteria* and vortexed for 30 seconds. Following this treatment, embryos were cut into ~1 mm lengths and placed under vacuum (~ 20 in. Hg) for 30 minutes before transfer to P5 medium (~40 embryos per plate). After 6 days cocultivation, embryo pieces were transferred to P5 plus 10 mg/l geneticin and 200 mg/l timentin. These embryo pieces were cultured in the dark under the same conditions as described for the production of secondary embryos (Eady et al. 1998). Cultures were transferred to fresh medium every 2 weeks. After 3-4 transfers, growing material was transferred to P5 plus 25 mg/l geneticin and grown for a further 8 weeks. During this time pieces of putative transgenic tissue which reached ~2 mm^2 were transferred to regeneration medium (Eady et al. 1998). Shoot cultures were maintained for 12 weeks and developing shoots were transferred to 1/2MS media (Murashige and Skoog 1962) plus 20 mg/l geneticin to induce rooting. Rooted plants were either transferred to 1/2MS plus 120 g/l sucrose to induce bulbing or transferred to soil in the glasshouse(12 h 12-23°C day, 12h 4-16°C night).

Analyses for transformation: For GFP expression, tissue was examined by observation under a fluorescence microscope (excitation 475 nm, emission 510 nm Haseloff et al.1997). Larger tissues with high levels of expression were observed using hand held "shirt pocket" fluorescent lanterns (Zelco industries inc., 630 So. Columbus Ave, Mt Vernon NY 10551-4445). *NptII* expression was determined by the ability of regenerating plantlets to form roots on 1/2MS containing geneticin.

DNA isolation was performed using a nucleon phytopure plant DNA extraction kit (Amersham Lifescience, Buckinghamshire, England). Southern analysis followed the method of Timmerman et al. (1993) and used PCR-amplified probes to confirm the presence of the *gfp* gene. Genomic DNA from the onions was digested with *HindIII*, which cuts once in the middle of the T-DNA.

Cytology: Chromosome counts were made from the root tips of 2 primary transformants and followed the procedure of Grant et al. (1984).

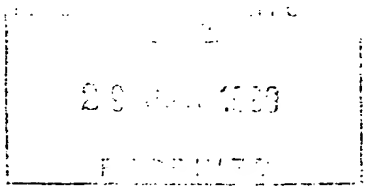
Example 1

Transformation and characterisation of primary transgenic tissue

After three days of cocultivation, single cells expressing GFP could be observed. Attempts to count cells expressing GFP after 5 days were abandoned as the variation within treatments and between embryo pieces was huge, with many embryo pieces showing no fluorescence and some exhibiting hundreds of fluorescing cells (Fig. 1a). In

the latter case, distinguishing between multicellular 'stable' transformation events and multiple adjacent single-celled 'transient' events was not possible. Thus, large biases in any measurement of initial transfer could have occurred. As an alternative, treatments were given an initial transfer rating: *** being excellent initial transfer (~20-30% of tissue pieces with >20 GFP positive cells per plate), ** represented average initial transfer (5-20% of tissue pieces with some GFP positive cells per plate, and * being poor initial transfer (< 5% of tissue pieces with GFP positive cells per plate) (Table 1). Contamination was a problem in many experiments. Often whole experiments (data not shown) had to be abandoned due to contamination, much of which probably arose from infected embryos.

After 2 weeks on selection medium, embryo pieces were screened for GFP expression and only pieces containing fluorescing cells were maintained (Fig. 1b). The vast majority of fluorescing cells died over the following four weeks. Some fluorescing cells divided into multicellular clusters of up to ~50 before their ability to fluoresce gradually faded. One interpretation of this was that the transformed cells were still reliant on surrounding non- transgenic cells, which died due to selection pressure and could no longer support the transgenic cells. The number of stable transgenic sectors arising from different plates within experiments varied from 0 to 21 and reflected the numerous parameters that affect the onion transformation process. Comparison between experiments was initially possible and ranged from * in contaminated samples to *** in non-contaminated samples. Indeed, lack of good initial transfer was often an early indication of



contamination. Comparison of variability in stable sector production was not possible between experiments as putative transgenic sectors were transferred to the wrong selective medium for 4 days, in 2 of the 3 successful experiments. Transfer to the wrong selection medium drastically reduced the survival of the fluorescing sectors. Eady et al. (1998) and Eady and Lister (1998ab) demonstrated that genotype, condition of the embryo, size of the embryo, cocultivation conditions and selection pressure all affect embryo survival. The combined effects of these parameters and their interaction with the transformation process will, until they can be controlled, continue to make the success of onion transformation susceptible to large variation.

Example 2

Regeneration

After 6 weeks of culture, tissue was transferred to a selective medium without timentin. No growth of *Agrobacterium* was observed in any of the cultures grown on this medium. Fluorescing sectors continued to grow on this media and after 2 transfers it was possible to isolate the first sectors free from non-fluorescing cells (Fig. 1c). As sectors became independent (Fig. 1d) they were transferred to regeneration medium. A few sectors still attached to non-fluorescing tissue were also transferred. On regeneration medium transgenic cultures responded in the same way as non-transgenic, embryo-derived cultures (Eady et al. 1998). Multiple shoots formed on many of the independent transgenic cultures (Table 1). However, some, particularly the slower growing or more friable dedifferentiated cultures, either failed to regenerate or produced highly

hyperhydric shoots that could not be transferred to the glasshouse. Up to 32% of stable sectors produced shoot cultures from which plants could be obtained (Table 1). These responses to regeneration are typical of those seen in non-transformed embryogenic cultures (Eady et al. 1998). Actively growing shoots were transferred to rooting medium containing geneticin. In the instances where non-fluorescing cells were also transferred to shoot media some shoots were produced that failed to root on geneticin. These did not fluoresce. All plants that formed actively growing roots on geneticin also fluoresced (Fig. 1f), indicating that in all instances the complete T-DNA was transferred. Fluorescence in the differentiated structures varied, with most fluorescence being seen in root tips. In shoots, strong fluorescence was limited to young shoots (Fig. 1e). However, GFP fluorescence in shoots was usually masked by red autofluorescence from the chlorophyll. The presence of GFP fluorescence in older leaves could sometimes be observed in the stomatal guard cells.

The multiple shoot cultures enabled clonal plants from independent transgenic events to be grown. This was particularly important as earlier attempts to exflask putative transgenic plants had failed (Eady and Lister 1998b). To date only 4 from 48 transgenic plants transferred to the soil have died. A total of 14 independent transformants have been transferred to the containment glasshouse (Fig. 1g).

Example 3

Analyses of transformants

Apart from fluorescence and growth on geneticin, transformation of onion plants was confirmed by Southern analysis, probing with the *gfp* gene (Fig. 2). As *HindIII* cuts the T-DNA only once it was possible to show copy number from the Southern analysis. Ten of the 13 transformants shown have single copies. The other 3 have 2 (lane 8), 3 (lane 18) and multiple copies (lane 7). Lanes 19 and 21 are from clonal shoots and, as expected, they show the same pattern. *EcoR1* digest and subsequent Southern analysis liberated an internal T-DNA fragment of ~ 900 bp.

Chromosome counts in the 2 primary transformants tested showed a diploid (2n=16) chromosome complement.

Discussion

We have developed a repeatable transformation system for onion. The regenerating primary transformants appear to be phenotypically normal. The GFP expression, as a visual selectable marker, enabled post transformation selection conditions to be optimised. It showed that the initial levels of geneticin required were lower than previously suggested for onion (Eady and Lister 1998a). The GFP marker has also proved useful in the selection of transgenic plants from other species that are difficult to transform (Vain et al. 1998). Now that selection conditions have been established, identification of transformants solely on their ability to root in selective media should be possible.

The regeneration system employed in this work and observations of non-transgenic cells being transferred with transgenic tissue would indicate the possibility of chimeric plants being produced. Although some non fluorescing shoots did arise, presumably from non-transgenic tissue, these could be rejected at the rooting stage. Fluorescing shoots were never observed to be chimeric suggesting that individual cells in the initial embryogenic tissue are, with the support of surrounding cells, totipotent.

This method of producing transgenic onions is repeatable and efficient. It takes a short time to produce transgenic plants and utilizes techniques that were developed to be cultivar independent.

It is to be understood that the scope of the invention is not limited to the described embodiments and therefore that numerous variations and modifications may be made to these embodiments without departing from the scope of the invention as set out in this specification.

For example, this described process of transformation can be used with any species within the *Allium* and is not limited to onions. Work has shown that the described process of transformation is genotype independent.

Table 1. Summary of 5 transformation experiments. * - poor, ** - average, *** - excellent initial transfer, see text for details. Numbers in brackets represent the

percentage of transformants from uncontaminated embryos (a - represents the stage and treatments which were transferred to the wrong selective media for 4 days).

Expt	N°. of embryos	% of embryos contaminated	Initial transfer	N°. of multicellular GFP tissue pieces		Independent plants	Positive Southern#
				4wk	8wk		
1	~400	100	*	-	-	-	-
2	~360	40	***	52(16)a	15(4.6)	2 (0.6)	1of 1 tested
3	~440	0	***	72 (16)	44 (10)	12 (2.7)	8 of 8 tested
4	~520	60	**	a	11 (2.4)	3 (0.7)	2 of 2 tested
5	~200	100	*	-	-	-	-

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Figure 1

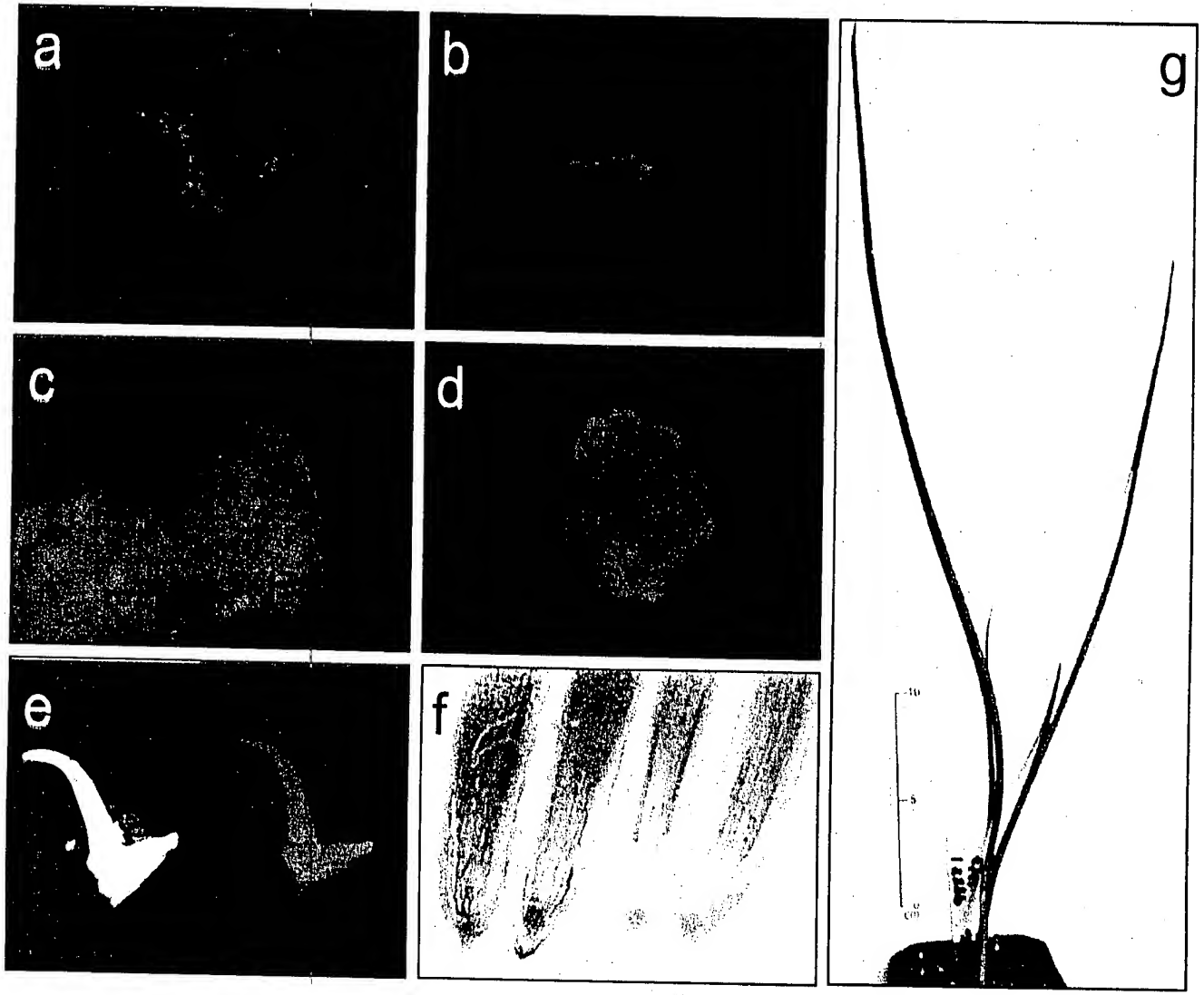
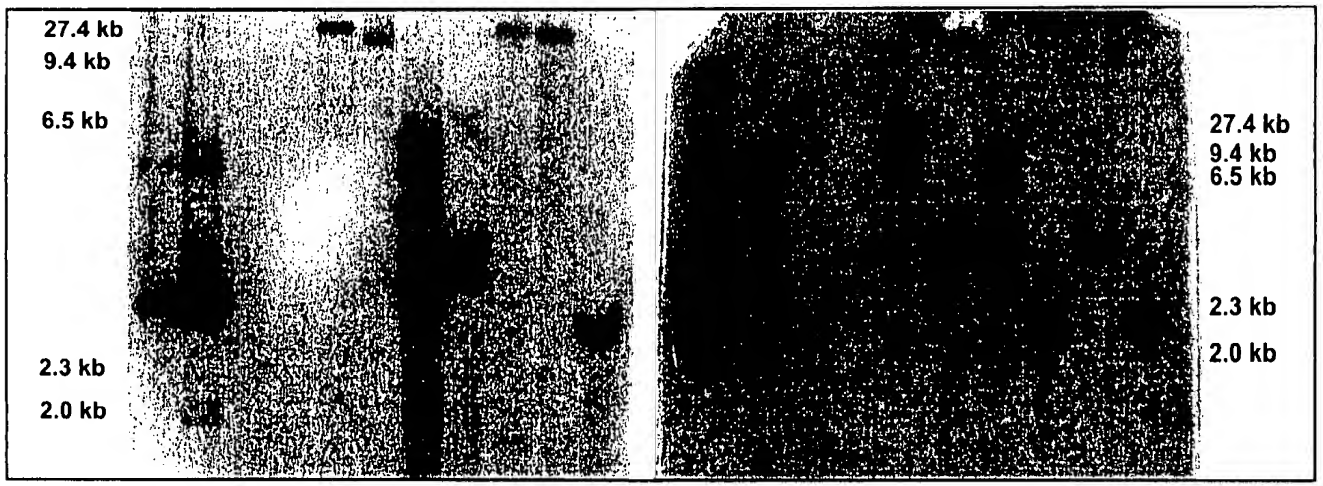


Figure 2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